10/528210
PCT/CA 03/ 01359
28 OCTOBER 2003 28.10.03

Office de la propriété intellectuelle du Canada
Un organisme d'Industrie Canada

Canadian Intellectual Property Office
An Agency of Industry Canada

Bureau canadien des brevets
Certification

La présente atteste que les documents ci-joints, dont la liste figure ci-dessous, sont des copies authentiques des documents déposés au Bureau des brevets.

Canadian Patent Office
Certification

This is to certify that the documents attached hereto and identified below are true copies of the documents on file in the Patent Office.

Specification and Drawings, as originally filed, with Application for Patent Serial No: **2,404,356**, on September 18, 2002, by **CANADIAN INOVATECH INC.**, assignee of Stephen R. Smith, Dr. Stewart J. Ritchie and Guopeng Zhang, for "Gram-Positive Antibacterial Composition and Method of Use".

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

Agent certificateur/Certifying Officer

October 22, 2003
Date

Canada

(CIPO 68)
04-09-02

OPIC CIPO

Gram-Positive Antibacterial Composition and Method for Use

Background of the Invention

Gram-positive bacteria are the cause of numerous animal diseases that affect livestock. One such bacterium is *Clostridium Perfringens* and causes disease in both swine and poultry. In poultry, *Clostridium Perfringens* causes Necrotic Enteritis ("NE"). In Swine, *Clostridium Perfringens* causes Clostridium Perfringens Enteritis ("CPE").

NE and CPE have been reported from most areas of the world and has been seen to have significant economic impact on the poultry and swine industry. NE as a domestic avian disease was first described in 1961 and the annual loss due to NE worldwide is estimated to be more than two billion US dollars.

Under favorable conditions inside the avian gut, this organism can multiply quickly and release toxins which cause gross lesions consisting of large areas of necrosis of the lining of the lower small intestine, also in caeca and liver in some cases. Intestinal stresses caused by dietary risk factors and coccidiosis etc. are considered the predisposing factor for the disease. The classical forms of NE tends to occur as outbreaks of severe diseases, principally affecting birds 2-5 weeks of age and characterized by an acute course of loss of appetite, depression, ruffled feathers, diarrhea, decreased rate of gain and sudden death. Mortality rate in untreated flocks can reach 10% or more.

A variety of antibiotics such as virginiamycin and bacitracin have been used to prevent or control *Clostridium Perfringens* related diseases. These antibiotics are administered to the animals by adding it to animal feed. However, antibiotic resistance does occur. In July 1999, the European community banned the use of feed-grade antibiotics, including virginiamycin and bacitracin. A similar action will soon be seen in the rest of the world due to the increasing public awareness of the negative impact of antibiotics on the environment and human health. The primary problem faced by poultry and swine producers in the absence of antibiotics is NE and CPE. Therefore there is a need to find

cost-effective alternatives to inhibit gram-positive bacteria in livestock and prevent gram-positive related diseases such as NE and CPE.

Description of the Invention

Lysozyme (EC 3.2.1.17, muramidase) naturally occurs in several mammalian secretions (milk, saliva, tears). In industry, it is extracted from hen egg white due to its abundance. It is a 14.6 kDa single peptide that can result in cell lysis by cleaving the β(1-4) glycosidic linkages between *N*-acetylmuramic acid and *N*-acetylglucosamine in the peptidoglycan layer of the bacterial cell. Lysozyme has an extremely high isoelectric point (>10) and consequently is highly cationic at neutral or acid pH. In solution, lysozyme is relatively stable at low pH and is active over the temperature range of 1°C to near boiling.

Lysozyme is effective against certain Gram-positive bacteria including *Clostridium* species. It has been used in the cheese industry as a bio-protectant for more than 20 years to prevent the butyric spoilage which causes the late blowing of semi-hard cheeses by *Clostridium Tyrobutyricum*.

In one embodiment of the invention, lysozyme is added to animal feed to control and prevent gram-positive bacteria related diseases in animals. The applicant has shown lysozyme to be very effective against gram positive bacteria such as *Clostridium Perfringens*. Figure 1 shows minimal inhibitory concentration ("MIC") plates for lysozyme against *Clostridium Perfringens*. Lysozyme shows efficacy for inhibiting *Clostridium Perfringens* at a dose of 200 ppm. Lysozyme can therefore be added to bird and swine feed in order to prevent NE and CPE respectively. Antibiotic usage costs approximately USD$3 to USD$5 per metric ton ("MT") of feed. In order to maintain efficacy at 200 ppm, lysozyme would cost USD$20/MT of feed. While the cost would increase, the invention has the advantage of using lysozyme as opposed to conventional anitbiotics.

In another embodiment of the invention, a composition containing lysozyme, albumen and an organic acid is added to animal feed to prevent gram-positive bacteria related diseases in animals. The applicant has shown that by adding albumen and an organic acid, such as citric acid, to lysozyme, there is an increased efficacy for inhibiting *Clostridium Perfringens* due to synergistic effects between lysozyme, albumen and citric acid. Citric acid works as an anti-oxidant and is synergistic with lysozyme. Keeping the gut of birds and swine acidic increases the overall effectiveness in inhibiting *Clostridium Perfringens*. Other native components of egg white or albumen, from which lysozyme is derived, work well in inhibiting numerous bacteria, yeasts, molds and virus as well as inhibiting many proteases. The toxins that are produced by *Clostridia* causing the symptoms of NE and CPE, are inhibited by the synergies of the egg white proteins.

Experimental data relating to the synergies between lysozyme, citric acid and albumen are illustrated in Figure 2. Figure 2 shows MIC plates of *Clostridium Perfringens* inoculated with a 100 times dilution of blend 2571B. Blend 2571B contains lysozyme, albumen and citric acid in a ratio of 50:150:50. As can be seen from the MIC plates, blend 2571B shows efficacy for inhibiting *Clostridium perfringens* at roughly 50ppm. Therefore although the relative amount of lysozyme has been decreased, the efficacy of the composition has increased as a result of the synergies between lysozyme, citric acid and albumen. It is important to note that although the effective inhibitory dose of blend 2571B is 50ppm, lysozyme represents only a fraction of that blend. Given the relatively low cost of albumen and citric acid as compared to lysozyme, blend 2571B would cost approximately USD$3/MT of feed to be effective in inhibiting *Clostridium Perfringens* at 50ppm.

In another embodiment of the invention, a composition containing both lysozyme and a lantibiotic is added to animal feed to prevent gram-positive bacteria related diseases in animals. The applicant has shown that by adding a lantibiotic, such as nisin, to lysozyme there is an increased efficacy for inhibiting *Clostridium Perfringens* due to synergistic effects between the lysozyme and the lantibiotic.

Experimental data relating to the synergies between nisin and lysozyme are illustrated in Figure 3. Figure 3 shows fractional inhibitory concentration ("FIC") plates of *Clostridium Perfringens* inoculated with a 100 times dilution of varying amounts lysozyme and nisin. Note that lysozyme is effective alone at 200ppm. However, by increasing the amount of nisin to 3ppm, only 8ppm of lysozyme is required to achieve a bactericidal effect on *Clostridium Perfringens*.

In another embodiment of the invention, a composition containing lysozyme, albumen, organic acid and a lantibiotic is added to animal feed to prevent gram-positive bacteria related diseases in animals. The applicant has shown that a composition containing a lantibiotic, such as nisin, lysozyme, albumen and an organic acid, such as citric acid has a high efficacy for inhibiting *Clostridium Perfringens* due to the synergies between lysozyme, nisin, albumen and citric acid.

Experimental data relating to the synergies between nisin, lysozyme, citric acid and albumen is illustrated in Figure 4. Figure 4 shows MIC plates of *Clostridium Perfringens* inoculated with a 100 times dilution of blends 250 and 270. Blends 250 and 270 contains lysozyme, nisin, citric acid and albumen in ratios of 33:17:50:150 and 50:20:50:150 respectively. The MIC plates show an effective dosage of this combination, against *Clostridium perfringens*, is roughly 20ppm. Therefore, by adding nisin, the effective dosage of has been decreased from approximately 50ppm (see blend 2571B) to 20ppm. A blend of lysozyme, nisin, citric acid and albumen is extremely cost effective at less than $1USD/MT of feed while maintaining efficacy for inhibiting *Clostridium perfringens* at 20ppm.

Minimal Inhibitory Concentration (MIC) of Lysozyme against *C. perfringens* IM248

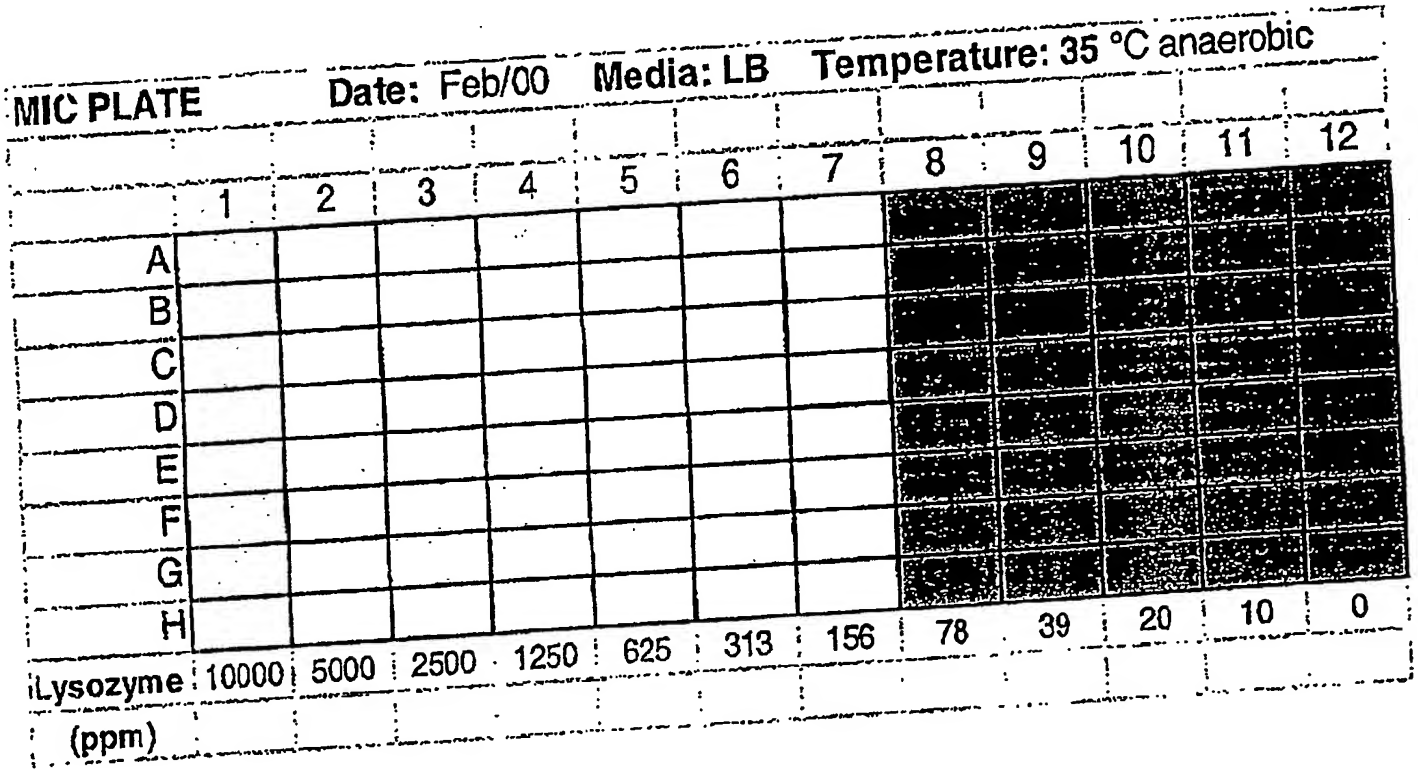


Figure 1

Figure 2

: PLATE Set up Date: July 3/02 Recording Date: July 5/02 Media: LB Temperature: 30°C

48 *Clostridium perfringens* inoculated with 10^{-3} dilution

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
o bacteria G												
o bacteria H												
(ppm)	10000	5000	2500	1250.0	625.0	312.5	156.3	78.1	39.1	19.5	9.8	0

nd 25718: Lyso/albumen/citric acid 50:150:50

C PLATE Date: Media: Temperature:°C

	1	2	3	4	5	6	7	8	9	10	11	12
(ppm)	100	50	25	12.5	6.3	3.1	1.6	0.8	0.4	0.2	0.1	0

IC PLATE Date: Media: Temperature: °C

	1	2	3	4	5	6	7	8	9	10	11	12
(ppm)	10000	5000	2500	1250.0	625.0	312.5	156.3	78.1	39.1	19.5	9.8	0

KIC PLATE Date: Media Temperature: °C

	1	2	3	4	5	6	7	8	9	10	11	12
(ppm)	10000	5000	2500	1250.0	625.0	312.5	156.3	78.1	39.1	19.5	9.8	0

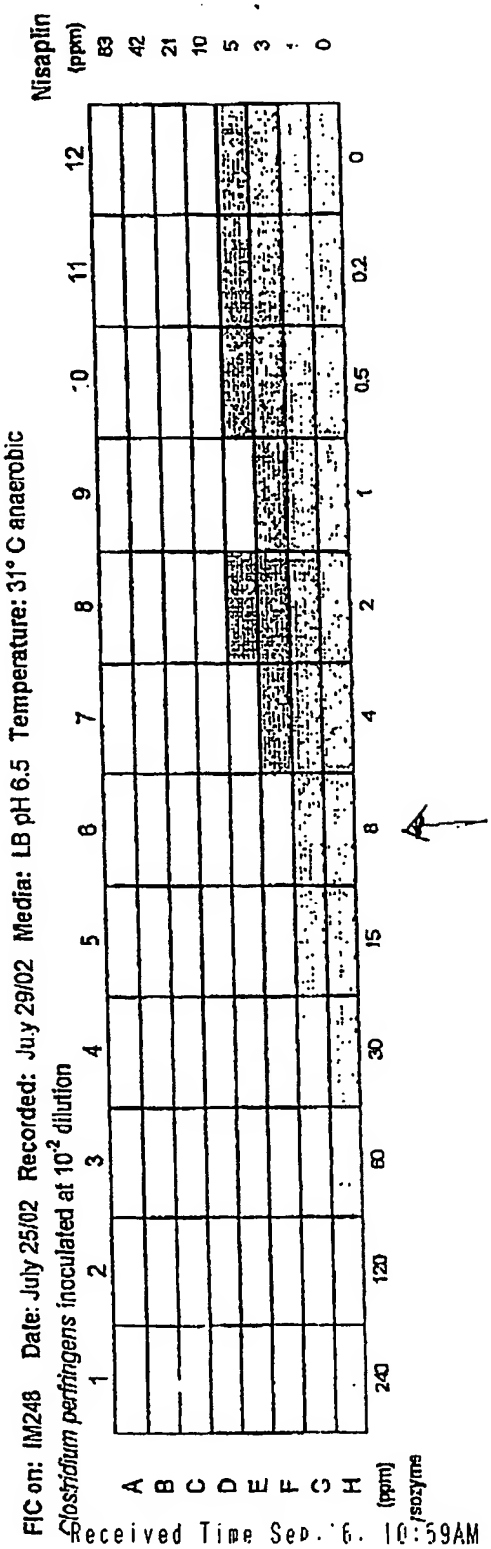
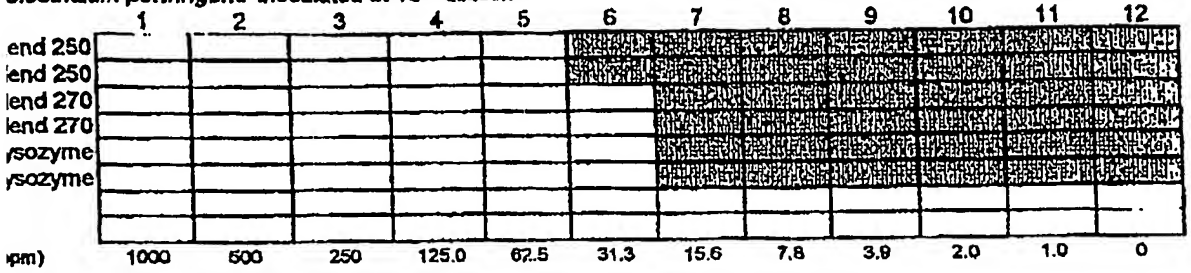


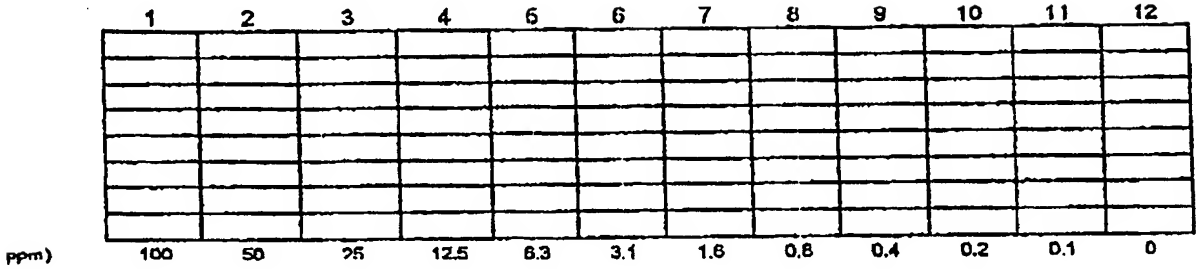
Figure 4

ATE Date: July 24/02 Recorded: July 29/02 Media: LB pH6.5 Temperature: 31°C anaerobic
Clostridium perfringens inoculated at 10^{-2} dilution



50: lysozyme/nisin/citric acid/albumen 33:17:50:150
70: lysozyme/nisin/citric acid/albumen 50:20:50:150

LATE Date: Media: Temperature:°C



LATE Date: Media: Temperature: °C

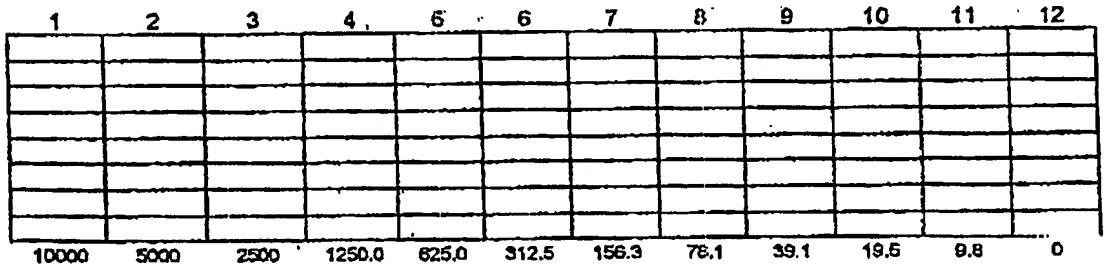


PLATE Date: Media Temperature: °C